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The hypothesis is that inactivation of Treg cells accompanied by Neu DNA vaccination will overcome tolerance in BALB NeuT mice and inhibit spontaneous mammary tumorigenesis or reject an established s.c. tumor. The anticipated tumor growth inhibition may be achieved at the risk of developing autoimmunity. Thyroiditis will be measured to indicate the level of risk. We further hypothesize that DNA vaccines encoding both Neu and GITR ligand will stimulate effector T cells via a conventional TCR interaction and inhibit suppressor activity via GITR signaling, thus inducing anti-tumor immunity without systemic Treg cell inactivation and the inadvertent induction of autoimmune diseases.

To test the hypothesis that anti-tumor but not autoimmunity can be induced by DNA vaccine encoding Neu-TM and GITRL, we will (A) construct and test DNA plasmids encoding NeuTM and GITRL and (B) perform in vitro and in vivo testing of pVIVO-NeuTM/GITRL. Toward sub-task B, we will (I) establish the read-outs for NeuTM DNA vaccination, including humoral and cellular immunity, (2) establish the read-outs for autoimmune response by measuring immune reactivity to mTg and inflammatory infiltration in the thyroid and (3) measure anti-Neu and anti-mTg reactivity in mice immunized with DNA encoding NeuTM/GITRL.

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## INTRODUCTION

The project was funded for two years for

Task 1 Test the hypothesis that anti-tumor but not autoimmunity will be induced by DNA vaccine encoding Neu-TM and DTA-1 scFv

- (A) Month 1-24 Construction and biochemical testing of DNA plasmids encoding NeuTM/DTAscFv
- (B) Month 1-24 In vitro and in vivo testing of pVIVO-NeuTM/DTAscFv
  - I. Establish the read-outs for NeuTM DNA vaccination, including antibody production and T cell response measured by ELISPOT.
  - II. Establish the read-outs for autoimmune response, including antibody and T cell reactivity to mouse thyroglobulin and inflammatory infiltration in the thyroid.
  - III. Measure anti-Neu and anti-mTg reactivity in mice immunized with DNA encoding NeuTM/DTAscFv.

The ligand for GITR, glucocorticoid-induced TNF receptor family member (TNFRSF18 or GITR), was cloned by Dr. Hermann Waldmann (Oxford University, England) after this project was approved for support1. GITRL may be superior to anti-GITR mAb DTA-1 in triggering GITR. Dr. Waldmann has provided us with the c-DNA of mouse GITRL. We will substitute DTA-1 scFv in the proposed construct with GITRL which has been shown to abrogate the activity of regulatory T cells and enhance the activity of effector T cells1. The Task is therefore revised to be:

Task 1 Test the hypothesis that anti-tumor but not autoimmunity will be induced by DNA vaccine encoding Neu-TM and GITRL

- (A) Month 1-24 Construction and biochemical testing of DNA plasmids encoding NeuTM/GITRL
- (B) Month 1-24 In vitro and in vivo testing of pVIVO-NeuTM/GITRL
  - I. Establish the read-outs for NeuTM DNA vaccination, including antibody production and T cell response measured by ELISPOT.
  - II. Establish the read-outs for autoimmune response, including antibody and T cell reactivity to mouse thyroglobulin and inflammatory infiltration in the thyroid.
  - III. Measure anti-Neu and anti-mTg reactivity in mice immunized with DNA encoding NeuTM/GITRL.

## BODY

**Objective 1A Construction and biochemical testing of DNA plasmids encoding NeuTM/GITRL**

Full length mouse GITRL in pMTF was provided by Dr. Herman Waldmann. The extracellular domain was amplified by PCR and cloned in pVIVO. Expression of recombinant mouse GITR and the ecd portion of GITR was tested by transient transfection of mouse mammary tumor cells D2F2, followed by staining with goat anti-

GITRL serum with PE conjugated $2^{nd}$ Ab. Figure 1 shows the detection of full length GITRL on the surface of transfected cells and the intracellular stain of GITRL-ecd.

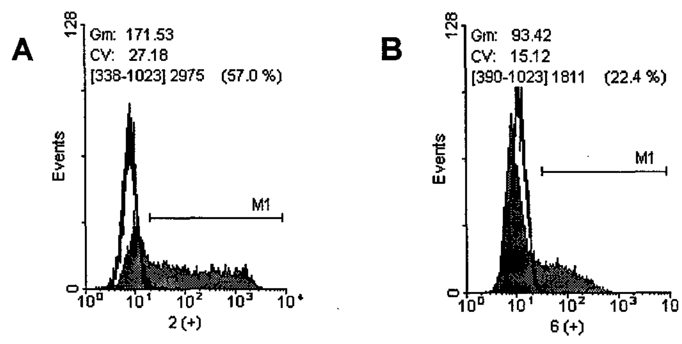


**Figure 1.** Expression of mouse GITRL and GITRL-ecd. D2F2 cells were transfected with pMTF-GITRL or pVIVO-GITRL-ecd. At 24 hrs after pGITRL transfection, cells were stained with goat anti-GITRL antiserum and PE conjugated $2^{nd}$ Ab before they were subjected to flow cytometric analysis (A). Cells transfected with pVIVO-GITRL-ecd were fixed, permeablized and stained to detect intracellular GITRL-ecd (B). Filled histograms represent cells stained with anti-GITRL antiserum. Open histograms were cells stained with control serum.

**Objective 1B-I Establish the read-outs for NeuTM DNA vaccination, including antibody production and T cell response measured by ELISPOT.**

To establish a test system for measuring antibody response to rat neu, BALB/c 3T3 cells (ATCC) were stably transfected with rat neu to establish 3T3/N cells. The mouse anti-rat neu mAb (IgG2a, clone 7.16.4), which recognizes an extracellular domain of rat neu protein (Oncogene Research Products, Cambridge, MA) was serially diluted and used to stain 3T3/N cells with FITC conjugated $2^{nd}$ Ab to establish a standard binding curve. Test sera was similarly tested and the concentration calculated by regression analysis as we previously reported2. Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson).

To assess anti-neu humoral and cellular immune responses in mice, BALB/c mice were inoculated with neu positive TUBO tumor and subjected to anti-CD25 treatment on days 1 and 3 after tumor inoculation to remove regulatory T cells. In anti-CD25 mAb treated mice, all mice developed tumors which started to regress when they were 15-180 $mm^3$ in size, and regressed completely by wk 11 (Fig. 2A), suggesting *in vivo* priming by a growing TUBO tumor. The course of tumor growth and regression was nearly identical whether anti-CD25 mAb was administered before or after tumor cell inoculation. Sera were collected at 5 and 9 wk following tumor cell inoculation and anti-neu antibody was measured by flow cytometry. As shown in Figure 2B, anti-neu antibody was not detected at wk 5 in the control group although palpable tumors were detected. The tumor volume exceeded 500 $mm^3$ at wk 9 (not shown), at which time $11.4 \pm 3.5$ μg/ml of anti-neu IgG were detected. Mice treated with anti-CD25 mAb before or after tumor cell inoculation displayed $11.7 \pm 8.6$ and $8.9 \pm 6.4$ μg/ml, respectively, of anti-neu IgG at wk 5. At wk 9, when the tumors were almost completely eliminated, these same mice had $16.7 \pm 9$ and $34 \pm 8.6$ μg/ml of anti-neu IgG, respectively ($P = 0.01$). Thus, antibody response can be reliably monitored with the flow cytometric analysis as in these mice (Fig. 2B).

To measure neu-specific T cell response, the spleen cells were isolated from mice which rejected TUBO tumors and incubated with the engineered 3T3/NKB cells which expressed neu, $K^d$ and B7.1 (CD80). IFN-γ producing cells were enumerated by ELISPOT assay after a 2-day culture using the ImmunoSpot analyzer (CTL, Cleveland, OH). Fig. 2C shows the result from a representative animal. There were $36 \pm 2$ neu-specific IFN-γ producing cells per $10^6$ spleen cells.

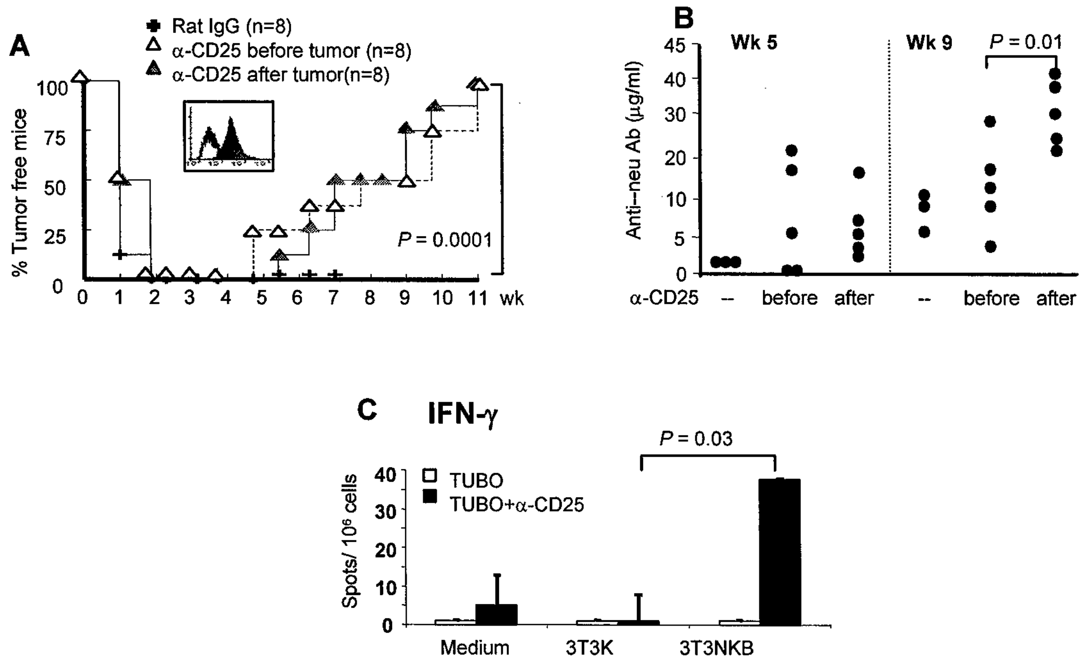


**Figure 2.** Anti-neu immune response in mice undergoing tumor regression. BALB/c mice were injected i.p with 0.5 mg anti-CD25 mAb either 5 and 6 days before or 1 and 3 days after mice received $2x10^5$ TUBO cells s.c. Control mice were treated with normal rat Ig or PBS. There were eight mice in each group. Tumor growth was monitored for 11 wk. (A) TUBO tumor growth in mice treated with anti-CD25 before (Δ) or after tumor cell inoculation (▲) or in untreated mice (+). The results are expressed as percentage of tumor-free mice. Expression of neu on TUBO cells (Insert) was verified by flow cytometry using mouse-anti-rat neu mAb (7.16.4, filled histogram) followed by PE-anti-mouse Ig, and compared to isotype controls (open histogram). (B) anti-neu IgG levels in mice treated with anti-CD25 before or after TUBO cell inoculation were measured by flow cytometry and calculated by regression analysis. (C) T cell reactivity was measured by ELISPOT after 48 hr incubation with 3T3NKB cells. Control wells received 3T3K cells or medium.

## Objective 1B-II Establish the read-outs for autoimmune response, including antibody and T cell reactivity to mouse thyroglobulin and inflammatory infiltration in the thyroid

Immune reactivity to mouse thyroglobulin (mTg) was induced by two time i.v. injection, one wk apart, with 40 ug mouse thyroglobulin (mTg) and 20 ug LPS. After 28 days, blood, spleens, and thyroids were removed for analysis. Serum antibodies to mTg were analyzed by ELISA. To analyze mTg-reactive T cells, spleen cells were cultured

with mTg *in vitro* for 5 days, and response to mTg measured by tritiated thymidine incorporation. Thyroids were removed into formalin, sectioned and stained with H&E. Percentage of thyroid infiltration and/or destruction was scored.

BALB/c mice (MHC class II H-$2^d$) resistant to EAT were depleted of regulatory T cells 10 days before treatment with mTg and LPS. As shown in Figure 3A, mice depleted of regulatory T cells and treated with mTg and LPS had significantly elevated anti-mTg antibodies, as compared to control mice ($OD_{405}$ 0.9 vs. 0.004; 1:2,500 dilution; $P = 0.003$). To measure mTg-reactive T cells, whole spleen cells were incubated *in vitro* with mTg. In mice depleted of regulatory T cells, proliferation of mTg-reactive T cells was significantly increased, compared to controls (Fig. 3B). The stimulation index was 25 in test vs. 14 in control mice. A significant increase in thyroid infiltration was associated with this elevated anti-mTg immune reactivity (Figure 3C). Therefore, anti-mTg humoral and cellular immunity can be reliably measured by ELISA and proliferation assays, respectively. In regulatory T cell depleted, EAT-resistant BALB/c mice, there is a significant increase in immune reactivity to mTg, which correlates with an increased thyroid infiltration and destruction.

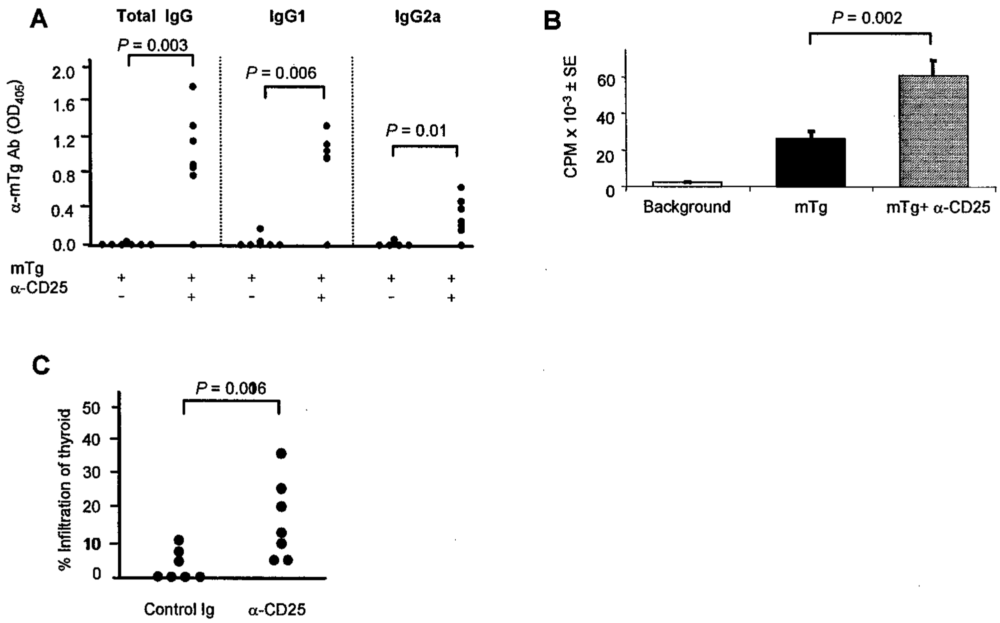


**Figure 3.** Enhanced anti-mTg immunity following Treg depletion. BALB/c mice were injected with 0.5 mg anti-CD25 mAb 4 days apart. On days 5 and 12 after the second injection, each mouse received i.v. 40 μg mTg and 20 μg LPS. On day 35, thyroids, sera and spleen cells were collected for evaluation. (A) mTg antibody measured by ELISA. Serum samples were tested at 1:2,500 dilution. (B) *In vitro* proliferative response to mTg. A total of $6 \times 10^5$ spleen cells were incubated in the presence of 40 μg/ml mTg for 4 days and [$^3$H]thymidine uptake was measured. (C) Infiltration of individual thyroids by mononuclear cells in histological sections was recorded. There were seven mice in each group.

## KEY RESEARCH ACCOMPLISHMENTS

1. Subclone GITRLecd and verify the proper expression of recombinant GITRL and GITRLecd.
2. Establish the read-outs for accessing humoral and cellular immunity to neu.
3. Establish the read-outs for accessing humoral and cellular immunity to mTg.

## REPORTABLE OUTCOMES

J.B. Jacob, Y.M. Kong, J.C. Flynn, D. Shim, J. Zelinski, and W.-Z. Wei Concurrent induction of anti-tumor immunity and autoimmunity – A new model for testing tumor immunotherapy and assessing thyroiditis induction. Proc. AACR. 2004

Jennifer B. Jacob, Yi-chi M. Kong, Olga Radkevich, Chella S. David, Ibrahim Mansoor, Daniel Snower, Wei-Zen Wei. Regulatory T cell status and MHC II haplotypes determine the balance between overcoming Her-2 tolerance by vaccination and inducing autoimmune thyroiditis. Proc. AACR, 2005.

## CONCLUSIONS

The project has proceeded according to plan. Full length GITRL cDNA has been obtained and should be superior to anti-GITR scFV as the triggering agent of GITR since this is the autologous molecule and can be administered repeatedly. The test systems for assessing both anti-neu and anti-mTg have been established. Vaccination studies are being initiated.

Reference List


1. Tone M et al., *Proceedings of National Academy of Science* early pub, (2003).
2. M. P. Piechocki, S. Pilon, WZ. Wei, *Journal of Immunological Methods* 259, 33-42 (2002).


## APPENDICES

J.B. Jacob, Y.M. Kong, J.C. Flynn, D. Shim, J. Zelinski, and W.-Z. Wei Concurrent induction of anti-tumor immunity and autoimmunity – A new model for testing tumor immunotherapy and assessing thyroiditis induction. Proc. AACR. 2004

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**2004 Annual meeting for American Association for Cancer Research**

*Proceedings of the American Association of Cancer Researchers – Abstract #2469*

Concurrent induction of anti-tumor immunity and autoimmunity – a new model for testing tumor immunotherapy and assessing induction of autoimmune thyroiditis.

**J.B. Jacob[1], Y.M. Kong[2], J.C. Flynn[2], D. Shim[1], J. Zelinski[1], G. Alsharabi[3], A.A. Giraldo[3], and W.-Z. Wei[1]**

Karmanos Cancer Institute[1] and Department of Immunology and Microbiology[2], Wayne State University, School of Medicine, Detroit, MI 48201; Division of Immunopathology[3], St. John Hospital and Medical Center, Detroit, MI 48236.

Induction of specific immune response has been associated with tumor rejection and autoimmune diseases and inhibition of regulatory T (Treg) cells can enhance both anti-tumor and autoimmunity, indicating the presence of cells with suppressor function. Here, we test if Neu-specific anti-tumor immunity can be amplified by modulating Treg cells and measure the concurrent induction of experimental autoimmune thyroiditis (EAT) in EAT-resistant BALB/c mice to establish a combined test system for developing cancer vaccines while minimizing autoimmunity. Her-2/neu is a defined tumor associated antigen (TAA) and our Her-2 DNA vaccine (E2A) encoding full length Her-2 with a.a. 753 K→A substitution is in a phase I clinical trial in patients with advanced breast cancer. EAT is a murine model of Hashimoto's thyroiditis wherein mice develop thyroid infiltration following immunization with mouse thyroglobulin (mTg). To test the effect of Treg cells on tumor growth, mice were depleted of $CD4^{+}25^{+}$ Treg cells by i.p. injection of ~0.5 mg α-CD25 mAb PC-61 at 3 and 1 day before they received $2.5x10^{5}$ TUBO cells s.c. TUBO is a cell line established from a spontaneous mammary tumor in a BALB/c mouse transgenic for transforming Neu. Mice treated with PC-61 displayed a significant reduction in lymph node resident $CD4^{+}CD25^{+}$ cells (13% on day 0 vs. 3% on day 7 following treatment). In parallel groups mice received 40 μg mTg and 20 μg LPS i.v. on days 5 and 12 with or without PC-61 treatment. All TUBO injected mice developed tumors in 5 wk. While tumors grew progressively in control mice, in mice treated with PC61 all tumors regressed completely by wk 9. Consistent with tumor regression, α-Neu antibody levels were increased in Treg cell depleted mice (18 μg/ml vs. 3 μg/ml). These results demonstrate *in situ* priming to TAAs that led to tumor rejection when Treg cells were depleted. Mice immunized with mTg were sacrificed on day 40 and EAT assessed by histologic examination of thyroids, *in vitro* lymphocyte proliferation to mTg, and α-mTg antibody production. Compared with mTg and LPS immunized mice, Treg depleted mice given mTg and LPS had increased thyroid infiltration (15% vs 3%), α-mTg antibodies ($OD_{405}$ 1.46 vs 0.39 at a dilution of 1:2500) as measured by ELISA, and mTg-responsive lymphocytes (stimulation index 25 vs 14). Therefore, modulation of Treg cells enhanced Neu-specific anti-tumor immunity and augmented EAT in resistant BALB/c mice. With this combined Her-2/neu and thyroiditis model, we will test new strategies for Her-2/neu specific immunotherapy while minimizing induction of autoimmune diseases. This work was supported by CA 76340 and DK45960.

**2005 Annual meeting for American Association for Cancer Research**

**Regulatory T cell status and MHC II haplotypes determine the balance between overcoming Her-2 tolerance by vaccination and inducing autoimmune thyroiditis**

*Jennifer B. Jacob, Yi-chi M. Kong, Olga Radkevich, Chella S. David, Ibrahim Mansoor, Daniel Snower, Wei-Zen Wei.* Wayne State University, Detroit, MI, Mayo Clinic, Rochester, MN, St. John's Hospital and Medical Center, Detroit, MI

Human ErbB-2 (Her-2) transgenic (Tg) mice were generated in C57BL/6 background ($H\text{-}2^b$) to express Her-2 as a self-antigen. Vaccination of Her-2 Tg mice to overcome tolerance would mimic that in patients. Three-time electro-immunization with pE2TM encoding the extracellular and transmembrane domains of Her-2 together with pGM-CSF induced only moderate to poor α-Her-2 Ab response. Depletion of $CD4^+25^+$ regulatory T cells (Treg) with α-CD25 mAb before the 1st immunization increased the level of α-Her-2 Ab from 1.5 ± 3.0 to 25 ± 14 μg/ml. Increased T cell response was demonstrated by IFN-γ ELISPOT assay. The benefit of enhancing α-Her-2 immunity by Treg depletion may, however, be accompanied by an increased risk of autoimmunity to other self-antigens. In addition to Treg status, MHC II haplotypes are the major determinants of susceptibility to autoimmune diseases. In particular, expression of human HLA-DR3 predisposes humans to autoimmune thyroiditis and DR3 transgenic mice to experimental autoimmune thyroiditis (EAT). To assess the effect of Treg status on autoimmunity in high and low risk MHC background, Her-2 Tg (Her-2/$IA^b$) mice were crossed with DR3/$IA^{null}$ Tg mice to generate (Her-2/$IA^b$xDR3)$F_1$ mice. Immune reactivity to mouse thyroglobulin (mTg) was measured after 16 i.v. injections with 40 μg mTg over 4 wks or after two time i.v. injections with 40 μg mTg and 20 μg LPS with a 1 wk interval. Thyroid infiltration accompanied by destruction of the follicles was observed in Her-2/$IA^b$xDR3 but not $IA^b$ mice that received mTg and LPS, showing that the additional DR3 transgene confers susceptibility to EAT. mTg specific Ab from Her-2/$IA^b$xDR3 and Her-2/$IA^b$ mice which received 16X mTg injections were measured by ELISA. At 1:12,500, the level was 1.2 ± 0.6 and <0.2 ($OD_{405}$), respectively. mTg specific T cell response measured by ELISPOT and T cell proliferation assays was significantly higher in Her-2/$IA^b$xDR3 mice. Therefore, expression of DR3 resulted in strong immune reactivity to mTg. The effect of down-modulating Treg on concurrent Her-2 and mTg reactivity in Her-2/ $IA^b$xDR3mice is being evaluated. Although Her-2/$IA^b$ mice are resistant to thyroiditis, depletion of Treg cells before the 1st mTg injection resulted in mTg Ab and IFN-γ producing T cells, comparable to those in Her-2/$IA^b$xDR3 mice. These results demonstrate that the immune system of $IA^b$ or Her-2/$IA^b$ mice can respond to mTg, but this activity is suppressed by Treg. With reduced Treg, these mice become more susceptible to thyroiditis. In conclusion, when down-modulating Treg, the balance between the efficacy of cancer vaccine and the risk of autoimmunity may be influenced by MHC II haplotypes. It would be prudent to evaluate new cancer immunotherapy strategies accordingly. (CA76340, DOD W81XWH-04-1-0546, DK45960, St. John's Hospital and Medical Center).